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㉚ **Tissue culture method for transformation of plant cells.**

㉛ Plant cells in tissue culture are more efficiently transformed in a method which involves treatment with a cytokinin followed by incubation for a period sufficient to permit undifferentiated cells in cotyledonary node tissue to differentiate into meristematic cells and to permit the cells to enter the phases between the G1 and division phases of development.

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## TISSUE CULTURE METHOD FOR TRANSFORMATION OF PLANT CELLS

## BACKGROUND OF THE INVENTION

1 This invention relates to the transformation of plant cells, and particularly to improved methods for the  
5 transformation of intact plant cells in tissue culture.

Much research in plant molecular biology is now directed to the improvement of plant varieties via use of recombinant DNA techniques. Historically, plant breeders used classical genetic techniques to identify, preserve and crossbreed varietal lines having desirable traits. More recently, new plant varieties were induced by chemicals or by radiation treatment to mutate plant cells which were then regenerated using  
10 tissue culture techniques. These random and unpredictable approaches have obvious drawbacks. By the use of recombinant DNA technology, specific genes producing specific proteins, such as those imparting insect resistance, may be introduced into a plant to produce a desired variety with a particular trait. One method for the introduction of recombinant DNA into plant cells is the microparticle bombardment method described by Sanford et al., Journal of Particle Science and Technology, 5:27-37, the disclosures of which  
15 are hereby incorporated herein by reference.

In the transformation of cells by microparticle bombardment introduction of recombinant DNA to impart a desired trait, it is important that the transformation be efficient, i.e., that large numbers of cells be transformed by the method, at least transiently, so that the function and effectiveness of the structural gene of interest and/or the regulatory sequences associated with the structural gene of interest can be evaluated  
20 by conventional analytical methods. In the past, efficiency of transformation of some cell lines, such as soybean tissue culture cells, has been undesirably low, so that gene products were not formed in sufficient amounts to be analyzed quantitatively by simple methods. Several different treatments were tried to increase the level of transient gene activity. These have included treatment with various plant hormones, use of different cultivars of soybeans, and use of different transformation vectors. Disadvantages with each  
25 of these approaches continued to be low transient gene activity and a high degree of variability from experiment to experiment.

## DISCLOSURE OF THE INVENTION

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It has now been determined that treatment of plant tissue culture cells prior to microparticle bombardment has a large effect on their susceptibility to transformation. In particular, in the transformation of excised soybean meristems, it has now been found that it is important to pretreat the cells in a medium  
35 containing a cytokinin, followed by an additional period of culturing after cytokinin treatment to allow the cells to become synchronized in their reproductive cycles and to reach a phase of optimal susceptibility to transformation.

"Cytokinin" is used herein as a generic term for the class of plant hormones which promote cytokinesis and shoot formation in cultured plant cells and serve functions similar to kinetin [6-(furfurylamino)purine] or zeatin [6-(4-hydroxy-3-methyl-2-butenyl)aminopurine]. This term thus also encompasses synthetic analogs of  
40 naturally occurring cytokinins, hundreds of which are known in the art. The most effective of these are generally the N<sup>6</sup>-substituted adenine derivatives. A preferred compound is benzylaminopurine (BAP).

The plant meristem tissue is generally treated with cytokinin for a period of from about 12 to about 24 hours. A 24 hour treatment is effective and convenient and is preferred. Longer treatment periods do not  
45 appear to further enhance the efficiency of transformation. A preferred method of treatment is to place the excised tissue in a medium containing the cytokinin. When BAP is used it is preferably incorporated in the medium at a concentration of from about 0.1 mg/L to about 10 mg/L. Lower concentrations of from about 0.1 to about 0.5 mg/L have been found to be useful in the transformation of sunflower tissues, while higher concentrations of from about 2 mg/L to about 10 mg/L have been found useful in the transformation of  
50 soybean meristem tissues. In general, the concentration should be sufficient for the cytokinin to exert its usual hormonal effects on the plant tissues.

While not intending to be limited by theory, it appears that the treatment with cytokinin in the method of this invention interrupts division of the cells of the primary apical meristem for a period sufficient for the undifferentiated cells located at the cotyledonary node to differentiate into meristematic cells. This occurs because normal apical dominance is effectively suspended, for a period of from about 48 to about 72 hours.

When this occurs, the other undifferentiated cells can become active and begin to differentiate, which would not otherwise occur. Thus, this treatment can be described as having the net effect of synchronizing the time at which the cells enter the G1 and the division phases of development, so that when the cells are subjected to microparticle bombardment, more of the cells are in the optimal phase for transformation.

5 Anatomical studies also show that the phenotype of the cells of the surface are changed such that meristematic cells which are capable of division are present at the outermost layers, which are accessible to the microparticle bombardment. In this method the plant tissues are manipulated in a way which synchronizes the cell division process among the cells of the target tissue and promotes its responsiveness and sensitivity to the transforming recombinant DNA.

10 Thus, following treatment, the meristem tissue is incubated for a period sufficient to permit undifferentiated cotyledonary node cells to differentiate into meristem cells, and preferably for a large number of the cells to enter the desired phases between the G1 phase and the division phase prior to bombardment. Most preferably, the differentiated meristematic cells will be in the S phase at the time of bombardment. Depending upon the species, this can take from about 12 hours to about 72 hours. In soybean meristem  
15 transformation, approximately 48 hours is required after treatment. In any event, however, this period can readily be determined for any selected species and tissues by including in the process a number of control specimens which can be sectioned and examined microscopically at regular intervals for the above-described changes (differentiation of cotyledonary node cells into meristematic tissue, entry of large numbers of cells into the G1, S and division phases) to determine the optimum time for bombardment.  
20 These phases of the cell cycle will be well known to and easily recognized by plant cell biologists. The G1 phase is the phase immediately prior to DNA replication. It is recognized by 1x amount of DNA within the cells. The S phase is the phase of DNA replication, wherein the amount of DNA within the cells doubles. The division (M) phase is the phase of physical reorganization of the genetic material and physical division of the cell into two daughter cells, each of which contains 1x amount of DNA. S phase can also be identified  
25 by the propensity of cells to incorporate radiolabeled nucleotides. The incubation is done using conventional conditions of medium, light, and temperature.

In a preferred embodiment of the method of this invention, meristems are dissected from imbibed soybean seeds and are then pretreated in a medium containing 5 mg/L benzylaminopurine (BAP) for 24 hours. Following BAP treatment, the meristems are placed on a 1/2 strength Murashige-Skoog (MS) base  
30 culture medium containing 1% sucrose for an additional 24 hours. On the third day (2 days after cytokinin treatment), the exposed meristematic area is treated with microparticle bombardment. The typical result of bombardment on day 3 after excision (day 2 after cytokinin treatment) is that expression is at least one order of magnitude greater than bombardment immediately after subculture or without cytokinin treatment.

This method offers advantages in transformation of soybean cells because it allows transformation of  
35 substantially any soybean variety, rather than only those cultivars, such as Peking, which are commonly known to be easily transformed but which are also known to have poor agronomic characteristics. In addition, since having greater numbers of cells at the appropriate phase of development for transformation is desirable in transformation of any plant species, this invention offers an improved method of transformation of other difficult to transform crop species such as maize (corn), wheat, canola, millet, alfalfa, sorghum  
40 and rice.

Having now generally described this invention, the same will be better understood by reference to the following specific example, which is included herein for purposes of illustration only, and is not intended to be limiting unless otherwise specified. All percentages herein are by weight unless otherwise indicated.

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### Example

On day 0 of the experiment, soybean seeds of a proprietary cultivar known as Xb35b (Pioneer Hi-Bred  
50 International, Biotechnology Research Department, 7300 N.W. 62nd Ave., Johnston, IA) were surface sterilized with a 10% v/v solution of Clorox in water and soaked overnight in a 0.25M sorbitol solution.

On day 1 of the experiment (the following morning), the unifoliate (pumules) and trifoliate primordia were excised and removed. These were arranged in a petri dish on MS base medium containing 5 mg/L benzylamino purine (BAP). On the same day, one group of explants were subjected to microparticle  
55 bombardment using the method described in Tames, U.S Patent Application Serial No. 351,075, filed May 12, 1989. The explants were bombarded three times with particles carrying the pPHI413 vector of Beach et al. (Pioneer Hi-Bred International Biotechnology Research Department) which carries a beta-D-glucuronidase (GUS) gene to provide a visual and analyzable transformation marker, and placed in a dark

room at 28° C.

On day 2, another group of excised embryos were bombarded in the same manner. These were placed on shoot regeneration medium (MS base medium with 1% sucrose) and incubated in light at 28° C.

On day 3, yet another group of explants were bombarded and incubated in the same manner as the second group.

On day 4, a transient GUS assay was performed on each of the groups using two meristems per sample. Results were as shown in the following tables:

Table 1

Bombardment Schedule						% GUS Protein (10 <sup>-4</sup> )		
Day 1	Day 2	Day 3	Total	N	N <sup>+</sup>	Min	Mean	Max
3x	-	-	3x	8	0	-	-	-
-	3x	-	3x	14	5	1.7	2.9	4.3
-	-	3x	3x	6	6	8.2	42.1	117.5
3x	3x	-	6x	8	1	3.5	3.5	3.5
3x	3x	3x	9x	2	2	2.1	2.1	2.7
N = total number of samples								
N <sup>+</sup> = number of samples with positive GUS assay								

These results suggest that the treatment method of this invention is more valuable than additional bombardments in producing transient transformation with high efficiency.

### Claims

1. A method for transforming the cells of a plant tissue in tissue culture via microparticle bombardment, comprising the steps of
  - a. treating the tissue with a cytokinin; and
  - b. incubating the treated tissue for a period sufficient to permit the cotyledonary node cells in the tissue to differentiate into meristematic tissue cells prior to bombardment.
2. A method according to claim 1 wherein the tissue is excised meristem tissue.
3. A method according to claim 2 wherein the meristem is excised from an imbibed seed.
4. A method according to any one of the preceding claims wherein the seed is a soybean seed.
5. A method according to any one of claims 1 to 3 wherein the seed is a corn, sorghum, wheat, sunflower, canola, alfalfa, millet or rice seed.
6. A method according to any one of the preceding claims wherein the cytokinin is benzylaminopurine.
7. A method according to claim 6 wherein the benzylaminopurine is incorporated in culture medium at a concentration of from about 0.1 mg/L to about 10 mg/L.
8. A method according to any one of the preceding claims wherein the tissue is treated for a period of from about 12 hours to about 24 hours.
9. A method according to any one of claims 1 to 7 wherein the tissue is incubated for a period of from about 24 hours to about 3 days after cytokinin treatment and prior to bombardment.
10. A method according to any one of the preceding claims wherein the differentiated meristematic cells are in phases from the G1 phase to the division phase prior to bombardment.
11. A method according to claim 10 wherein the differentiated meristematic cells are in the S phase at the time of bombardment.



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# EUROPEAN SEARCH REPORT

Application Number

EP 90 31 1229

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-0 301 749 (AGRACETUS) * Example 9 * - - - -	1-4,6-8	C 12 N 15/87 C 12 N 15/82
A	WO-A-8 905 859 (UPJOHN) * Page 5; page 6, lines 15-25; page 8, lines 19-22 * - - - -	3	
A	CIBA FOUNDATION SYMPOSIUM, no. 137, APPLICATIONS OF PLANT CELL AND TISSUE CULTURE; SYMPOSIUM, Kyoto, 20th - 22nd October 1987, pages 144-163; I. POTRYKUS: "Direct gene transfer to plants" * Page 152, line 27 - page 154 * - - - -	10,11	
A	UCLA SYMP. MOL. CELL. BIOL., NEW SER., vol. 129, 1990, pages 21-34, PROCEEDINGS OF A UCLA SYMPOSIUM, Park City, Utah, 1st -7th April 1989, PLANT GENE TRANSFER, Wiley-Liss., Inc., New York, US; J. CAO et al.: "Transformation of rice and maize using the biolistic process" * Page 22, last 4 lines - page 23, paragraph 1 * - - - -	5	
A	JOURNAL OF CELLULAR BIOCHEMISTRY, Suppl. 13D, 27th March - 7th April 1989, page 266, abstract no. M 143, Alan R. Liss, Inc., New York, US; J. OARD et al.: "High velocity microprojectile delivery of foreign genes and their expression in apices of Triticum aestivum" * Abstract no. M 143 * - - - -	5	TECHNICAL FIELDS SEARCHED (Int. Cl.5)  C 12 N
A	PLANTA, vol. 167, no. 4, 1986, pages 473-481, Springer-Verlag; U.B. BARWALE et al.: "Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis" * Page 479, table 8 * - - - - -	1,4,7	
The present search report has been drawn up for all claims			
Place of search  The Hague		Date of completion of search  21 January 91	Examiner  MADDOX A.D.
<b>CATEGORY OF CITED DOCUMENTS</b> X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention		E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons ..... &: member of the same patent family, corresponding document	